

Claims

1. A method for analyzing methylation at one or more CpG positions to be analyzed in a nucleic acid sample, comprising:
 - a. converting unmethylated cytosine bases in the nucleic acid sample by treatment with an agent to uracil or another base that is dissimilar to cytosine in terms of base pairing behavior;
 - b. amplifying one or more nucleic acids of the treated sample in an amplification reaction, wherein at least two oligonucleotide primer pairs are provided for every CpG position to be analyzed, one of which primer pairs hybridizes preferentially in the case where the CpG to treated nucleic acid was methylated in the original sample before conversion, and further wherein the other of which primer pairs hybridizes preferentially in the case where the CpG to treated nucleic acid was unmethylated in the original sample before conversion;
 - c. detecting the amplicates formed in the polymerase reaction in a quantifiable manner; and
 - d. determining the degree of methylation in at least one CpG position of the nucleic acid sample.
2. The method of claim 1, wherein the converting agent is bisulfite or a compound thereof.
3. The method of claim 1, wherein amplification is carried out by a polymerase enzyme.
4. The method of claim 1, wherein the amplicates formed from each primer pair differ from those formed by another primer pair in at least one of length, sequence, and a detectable label.
5. The method of claim 4, wherein the detectable label is selected from a group consisting of fluorescence labels, mass labels, and radioactive labels.
6. The method of claim 1, wherein detecting the amplicates is carried out by means of mass spectrometry.
7. The method of claim 1, wherein detecting the amplicates is carried out by means of a real time technique.
8. A method for the analysis of the methylation status of one or more CpG dinucleotides within a nucleic acid sample, comprising:
 - a. in the nucleic acid sample, converting cytosine bases that are unmethylated at the 5-position by treatment with a converting agent to uracil or another base which is dissimilar to cytosine in terms of base pairing behavior;
 - b. amplifying one or more nucleic acids of the treated nucleic acid in a polymerase enzyme reaction by means of at least two primer oligonucleotide

- pairs, wherein one primer pair amplifies a reference sequence and the other primer pairs are methylation specific primers, and further wherein the amplificates formed from each species of primer pairs differ respectively in at least one of length, sequence, and a detectable label selected from a group consisting of fluorescence labels, mass labels, and radioactive labels;
- 5 c. detecting the amplificates formed from the primer pairs;
- d. measuring the amounts of the amplificates formed from each primer pair; and
- e. determining the degree of methylation at each analyzed CpG position.
9. The method of claim 8, wherein the converting agent is bisulfite or a compound thereof.
- 10 10. The method of claim 8, wherein the detection of amplificates is carried out by one of mass spectrometry and a real time technique.
11. The method of claim 10, wherein detection by mass spectrometry is carried out by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or electron spray mass spectrometry (ESI).
- 15 12. The method of claim 10, wherein detection by real time is carried out by means of one or more methods taken from the group comprising Real-Time PCR™ assay, TaqMan™ assay, LightCycler™ assay.
13. The method of claim 8, wherein at least three pairs of primers are used in the polymerase reaction, one of which primer pairs is a reference primer pair that amplifies a non-methylated sequence that acts as a reference sequence.
- 20 14. The method of claim 13, wherein the reference primer does not contain a CpG dinucleotide and does not contain a TpG dinucleotide.
15. The method of claim 13, wherein the primer pairs that do not amplify the reference sequence include one or more of CpG, TpG, and CpA dinucleotides.
- 25 16. The method of claim 13, wherein the amplificate synthesized from each primer pair is compared to the amplificate from the other primers and to the amount of amplificate from the reference primer.
17. The method according to claim 13, wherein determining the degree of methylation is carried out by determining the amount of each amplificate from each primer pair relative to the amount of amplificate formed from the reference primer pair.
- 30 18. The method of claim 13, wherein the amplificates are modified in such a manner that they become similar to peptides.
19. A method for the analysis of the methylation status of one or more CpG dinucleotides within a nucleic acid sample, comprising:

- a. converting cytosine bases that are unmethylated at the 5-position by treatment with a converting agent to uracil or another base that is dissimilar to cytosine in terms of base pairing behavior;
- b. amplifying one or more nucleic acids of the treated nucleic acid and of one or two reference samples in a polymerase enzyme reaction by means of one or more methylation specific primer oligonucleotide pairs, wherein the amplificates formed from each species of primer pair differ respectively in at least one of length, sequence, and a detectable label selected from a group consisting of fluorescence labels, mass labels, and radioactive labels;
- c. detecting the amplificates formed from the primer pairs within each sample
- d. measuring the amounts of the amplificates formed from each primer pair in each of the samples; and
- e. determining the amount of methylation within the treated sequence by determining the amount of amplificate formed within the treated sample relative to the amount of amplificate formed within the reference sample or samples for each primer pair.
20. The method of claim 19, wherein the converting agent is bisulfite or a compound thereof.
21. The method of claim 20, wherein the reference sample or samples is one of a fully methylated version of the target nucleic acid to be analyzed and a fully unmethylated version of the target nucleic acid to be analyzed.
22. The method of claim 20 wherein the detectable labels are selected from the group consisting of fluorescence labels, mass label, radioactive labels.
23. The method of claim 20, wherein detecting the amplificates is carried out by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).
24. The method of claim 20, wherein detecting the amplificates is carried out by means of one or more methods taken from the group comprising Real-Time PCR™ assay, TaqMan™ assay, LightCycler™ assay.
25. A plurality of oligonucleotide primer pairs for the determination of the degree of methylation at one or more CpG positions in a nucleic acid sample, wherein the oligonucleotide primer pairs are capable of distinguishing between methylated and non-methylated nucleic acid in the sample after modification by bisulfite treatment, and further wherein a first primer pair hybridizes preferentially to a modified nucleic acid that was methylated in the nucleic acid sample in the sequence the primer is hybridizing

- to, and a second primer pair binds preferentially to modified nucleic acid that was methylated in the nucleic acid sample in the sequence the primer is hybridizing to.
26. The primer pairs of claim 25, including at least one reference primer pair that is methylation insensitive.
- 5 27. The primer pairs of claim 25, wherein the amplicates synthesized from all species of primers are comparable to each other and differ according to at least one of length, sequence and detectable label and are thereby differentially detectable and quantifiable.
28. The primers of claim 25, wherein the amount of amplicate from each primer pair is compared to the amount of amplicate synthesized from the reference primer pair.
- 10 29. A kit providing for analysis of the methylation status of one or more CpG dinucleotides within a nucleic acid sample, comprising apparatus including a plurality of segments, including at least a first segment that contains an agent for converting unmethylated cytosines to another nucleotide base within the nucleic acid sample, a second segment that contains at least two oligonucleotide primer pairs that hybridize with a target
- 15 polynucleotide sequence and amplify CpG containing nucleic acid, one of which primer pairs hybridizes preferentially to converted nucleic acid that was methylated in the original sample in the sequence the primer is hybridizing to, and a second of which pairs hybridizes preferentially to converted nucleic acid that was unmethylated in the original sample in the sequence the primer is hybridizing to, and instructions for carrying out the
- 20 conversion and amplification, for detecting the amplicates formed in the polymerase reaction in a quantifiable manner and for determining the degree of methylation in at least one selected segment of the nucleic acid sample.
30. The kit of claim 29, further comprising at least one primer pair that either 1) amplifies a non-methylated sequence that acts as a reference sequence, or 2) is methylation
- 25 insensitive.